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Induction of somatic embryogenesis in different varieties of sugarcane (*Saccharam officinarum* L.)

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This investigation for the first time highlights TDZ induced callus formation, somatic embryogenesis and plant regeneration using leaf explants of different varieties (Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44) of sugarcane (*Saccharam officinarum*). Leaf explants cultured on the full strength inorganic salts Murashige and Skoog (1962) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ induced embryogenic tissue after 3-4 weeks. The percentage of somatic embryogenesis was not similar in all the tested varieties of sugarcane. This study indicates a high embryogenic potential of leaf explants of tested sugarcane varieties, and also opened up the possibility for large-scale clonal propagation of sugarcane for the genetic improvement programmes.

Key words: Belgaum, clonal propagation, Karnataka, leaf tissue, somatic embryogenesis.

Abbreviations: ABA - abscisic acid; MS - Murashige and Skoog medium; 2,4-D- 2, 4-dichlorophenoxy acetic acid ;TDZ- Thidiazuron.

Sugarcane (*Saccharam officinarum* L.) is one of the commercially the most important cash crops, accounts for nearly 70% of sugar production worldwide (Lakshmanan, 2006). Sugarcane is a highly polyploidy plant (2n= 36-170) grown in different parts of the world from the tropics to subtropics. The sugar industry in India plays a vital role towards socioeconomic development in the rural areas by mobilizing rural resources and generating higher income and employment opportunities (Murthy, 2010). India is the world's largest sugar consumer (Murthy, 2010). Molasses is the chief by-product of sugar industry and is the main raw material for alcohol production and alcohol-based industries in India (Murthy, 2010). The second by-product of sugar industry is bagasse, which is the fibrous material left over after crushing. Sugarcane bagasse is the chief source of power in the sugar mills. This is also being used as a raw material in the paper industry. In most sugar mills, cogeneration of power, using bagasse as fuel is considered feasible (Murthy, 2010). It has been estimated that about 3500 MW power can be generated annually without extra fuel and with investment much less than that required for generating the same through thermal power plants (Murthy, 2010). The third by-product of sugar industry is press mud, which contains many plant nutrients

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and could be an important source of organic matter, major and micro-nutrients (Murthy, 2010).

Being a highly ploidy, low fertility, large genome and complex environmental interactions make conventional breeding and genetic studies difficult for this crop (Manickavasagam *et al.* 2004). Conventional propagation of sugarcane in India is by vegetative means. However, these methods suffered from low propagation rates, expensive labor, time consuming and potential transmission of pathogens from the seed cane to the subsequent crop limits the efficiency of this method (Lakshmanan, 2006). Therefore, application of plant tissue culture techniques provides an alternative method for the crop improvement. Somatic embryogenesis is the production of embryo like structures under *in vitro* conditions under the influence of external growth hormones (Konar and Nataraja, 1965; Nataraja and Konar, 1970; Feher *et al.* 2003; Malabadi *et al.* 2009; Malabadi *et al.* 2011ab; Malabadi *et al*. 2010). The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants (Konar and Nataraja, 1965; Malabadi *et al.* 2011; Malabadi *et al.* 2010). Here, we report a protocol for the induction of somatic embryogenesis using young leaf rolls under the influence of TDZ and 2, 4-D. This protocol is very simple and reproducible and can be used for the production of transgenic plants for the genetic improvement of sugarcane.

Materials and methods

Initiation of embryogenic tissue

Fresh plant leaf material of sugarcane *Saccharam officinarum* varieties Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44 were harvested from 7-month-old field grown mother plants from Belgaum, Karnataka state, India. The leaf material was first washed and immersed (5min) in sterile distilled water for 3 to 4 times to get rid of dust particles. They were surface decontaminated sequentially with 0.1% streptomycin (1 min), 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma-Aldrich, St. Louis, USA), and thoroughly rinsed with sterilized double distilled water. Leaf sections were cultured on Murashige and Skoog, (1962) basal medium with 3.0% sucrose, 0.7% agar, 0.5 gl^{-1} *myo*-inositol, 1.0 gl^{-1} casein hydrosylate, 0.5 gl^{-1} L-glutamine, 250 mgl^{-1} peptone, 0.2 gl^{-1} *p*-aminobenzoic acid, and 0.1 gl^{-1} biotin, all purchased from Sigma. The medium was supplemented with a range of thidiazu-ron (TDZ) concentrations (0.45, 2.27, 4.54, 9.08 and 11.35 µM), and 2, 4-dichlorophenoxy acetic acid (2, 4-D) at a concentration of 4.52 µM singly and in combination without any other growth hormones. The cultures were raised in 25 mm × 145 mm glass culture tubes (Borosil, Mumbai, India) containing 15 ml of the above basal medium under cool white fluorescent light (Mysore lamps, India) at 100 µmol m^{-2} s^{-1} and 25 ± 3°C with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. Nutrient media without TDZ and 2, 4-D served as the control. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. Both L-glutamine, and TDZ were filter sterilized (Whatman filter paper, pore size = 0.45 µm; diameter of paper = 25 mm) and added to the media after autoclaving when the medium had cooled to below 50°C.

All the tissue cultures of *Saccharam officinarum* varieties Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44 were examined for the presence of different developmental stages of somatic embryos by morphological and cytological observations of callus. The cultures showing different stages of cell division were identified by microscopic observation. The callus was subcultured on the initiation medium for further 2 weeks for the better development of embryogenesis. The full strength inorganic

salts Murashige and Skoog, (1962) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ (induction medium) was used as an effective induction medium for producing the embryogenic tissue. On the other hand the callus without pro-embryonic cell divisions was considered as non-embryogenic. Non-embryogenic tissue was separated immediately from the rest of the tissue to avoid the overgrowth of the tissue. The efficiency of plant growth regulators and their concentrations were analyzed on the basis of visual observation (callusing percentage, percentage of explants forming embryogenic tissue, callus growth and callus necrosis). The ineffective treatments were discontinued.

Maintenance of embryogenic tissue

The embryogenic tissue of *Saccharam officinarum* varieties Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44 showing various developmental stages of somatic embryos was maintained on full strength inorganic salts MS (Murashige and Skoog, 1962) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ for the proliferation of callus (maintenance medium). The embryogenic tissue was subcultured for every 2 weeks. All the cultures were maintained under a cool white fluorescent light (100µmol m^{-2} s^{-1}) at 25±3°C with a relative humidity of 55-60%. The percentage of cultures showing somatic embryogenesis has been recorded.

Partial desiccation

For maturation, embryogenic tissue clumps of each of *Saccharam officinarum* varieties Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44 were partially desiccated. One gram fresh weight of tissue of each embryogenic line were transferred to sterile empty Petri dishes (60 mm diam.) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles) (Malabadi and Nataraja, 2006). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 hr to obtain the desired extent of desiccation (Malabadi *et al.* 2004c; Malabadi and van Staden, 2005ab; Malabadi and Nataraja, 2006). After desiccation, the partially desiccated tissue of each embryogenic line was transferred to maturation medium to induce further embryo development.

Somatic embryo maturation

The partially desiccated embryogenic tissue of *Saccharam officinarum* varieties Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44 were transferred to maturation medium to induce cotyledonary embryo development. The full strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium supplemented with 3.0% sucrose, 5 µM ABA and 0.8% agar (maturation medium) was adopted for this purpose. All the cultures were again maintained in the dark for 2 weeks. Microscopic observation was conducted to ensure the development of somatic embryos. The total number of somatic embryos produced after 2 weeks on maturation medium per one gram fresh weight of embryogenic tissue was recorded.

Germination and recovery of plantlets

After maturation, the somatic embryos were taken from the cultures for germination. The germination medium used was half strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium with 0.7% agar without any growth regulators (germination medium). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlet was based on the presence of an epicotyl. After 4 weeks on germination medium, the plantlets were directly transferred to vermiculite. Plantlets were placed in a growth room under a 16 hr photoperiod (50µ mol m^{-2} s^{-1}) for hardening. Somatic embryo proliferation in terms of root,

shoot development, plant conversion was recorded.

Statistical analysis

In all the above experiments, each culture tube received a single explant. Each replicate contained 20 cultures and one set of experiment is made up of 2 replicates (40 leaf sections were cultured for one set of experiment for *Saccharam officinarum* varieties Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44). All the experiments were repeated 3 times. Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's multiple range test (a=0.05) following ANOVA. All statistical analysis was performed using the SPSS statistical software package version 13.0.1.1 Microsoft Windows.

Results and Discussion

In the present investigation, the leaf explants induced callus after 2-4 weeks of culture on the full strength inorganic salts Murashige and Skoog, (1962) basal medium supplemented with 4.52 µM 2, 4-D and 4.54 µM TDZ (induction medium) in all the tested varieties (Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44) of *S. officinarum*. The callus growth was very slow and covered the entire surface of the leaf explants after 5 weeks. Embryogenic areas were clearly visible from the rest of the callus by their globular and glazy appearance and emerged as distinct white structures (Figure-1.1). In a control study, the leaf explants did not promote callus formation. Leaf explants remained green for three weeks and eventually turned brown and necrosed. Table-1 shows the embryogenic response of leaf-explants of all the tested varieties (Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44) of *S. officinarum* scored after 8 weeks on the initiation medium. The frequency of embryogenic tissue formation of all the tested varieties (Co94032, CoC671, Co86032, SNK754, SNK61 and SNK44) of *S. officinarum* was varied from each other (Table-1). The highest percentage of induction of somatic embryogenesis was noticed in a variety Co86032- 85.0±3.0a, and the lowest percentage of somatic embryogenesis was recorded in a variety SNK61-29.0±0.5a (Table-1, 2). All the tested varieties of sugarcane failed to induce embryogenic tissue on the higher concentration (11.35 µM) of TDZ. On the other hand lower concentration of TDZ (0.45, 2.27 µM) has induced very lower percentage of embryogenesis in all the tested varieties of sugarcane (Table-1). Therefore, 4.52 µM 2, 4-D and 4.54 µM TDZ are the optimum concentrations for the induction of embryogenic tissue in all the tested varieties of *S. officinarum.*

The embryogenic tissue was well maintained on the maintenance medium (Figure-1. 2). Microscopic observation of callus revealed different stages (2, 4, and 8 celled) of active cell division confirming the somatic embryogenesis on the maintenance medium in all the tested sugarcane varieties (Figure-1.2). Somatic embryogenesis occurred in the presence of TDZ in combination with 2,4-D, whereas it was never observed in explants cultured on control lacking hormones (Table-1). The partially desiccated embryogenic tissue was subcultured on maturation medium for further development of somatic embryos. In our present study, partial desiccation treatment has improved and enhanced the somatic embryo formation as reported earlier in many plant species (Malabadi *et al.* 2004c; Malabadi and Nataraja, 2006; Malabadi and van Staden, 2005ab; Suprasanna *et al.* 2008). This also confirms the previous findings of Desai and co-workers (2004), who has successfully induced and enhanced plant regeneration in sugarcane by the application of partial desiccation.

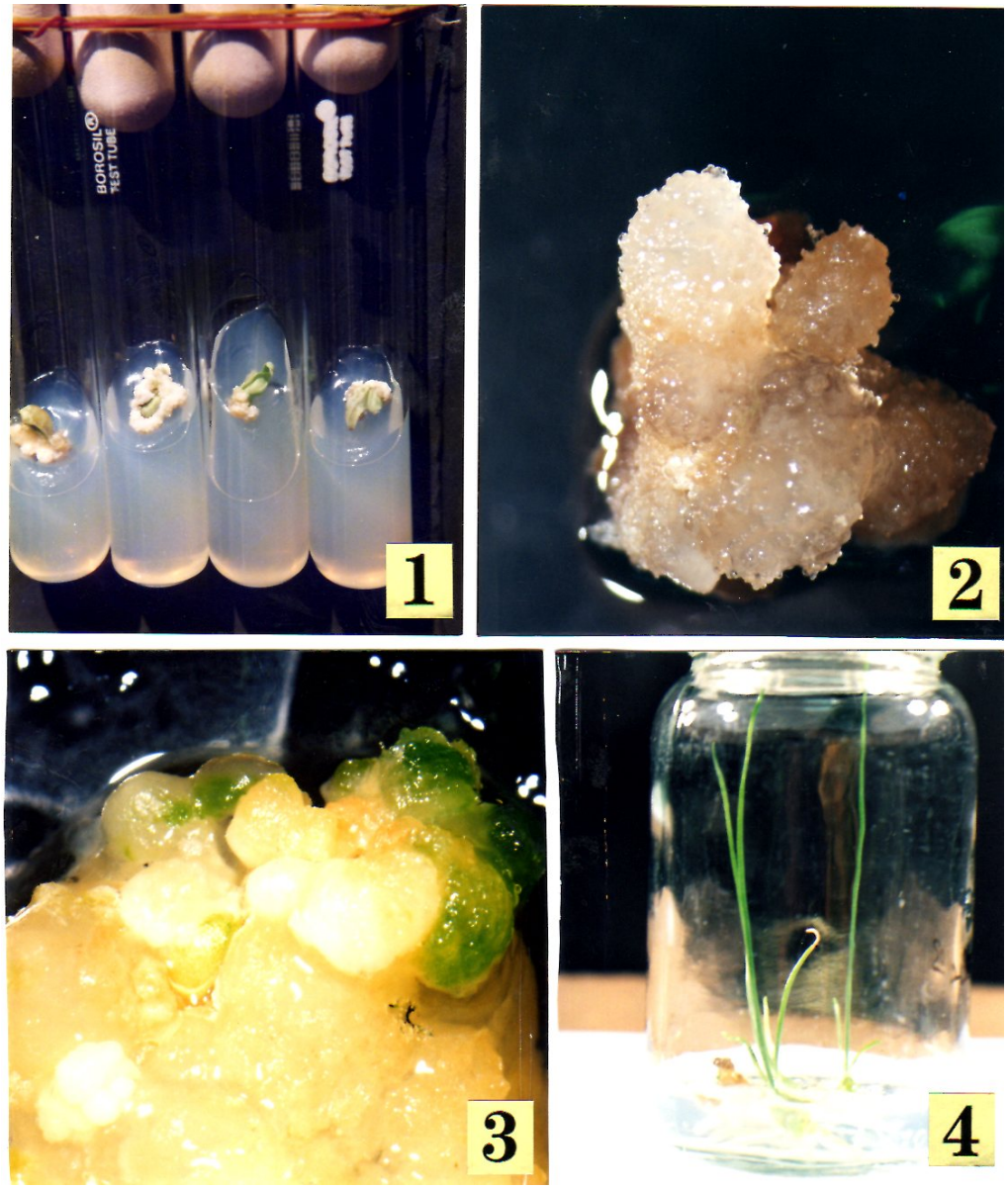

Figure. 1: TDZ induced somatic embryogenesis and plant regeneration in sugarcane variety Co86032. (1) Leaf explants showing proliferation of callus on initiation medium (10mm=3.90 mm). (2) Proliferation of embryogenic callus on maintenance medium (10mm=8mm). (3) Various developmental stages of somatic embryos seen under the microscope on maturation medium. (4) Germination of somatic embryos showing somatic emblings (10mm=3 mm).

On the other hand, the embryogenic tissue without partial desiccation resulted in the delayed maturation of somatic embryos in all the tested varieties of sugarcane lines. Therefore, partial desiccation treatment has boosted the formation of somatic embryos in all the tested varieties of sugarcane in our present study. All the cultures were again maintained in the dark for 2 weeks. The full strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium supplemented with 3.0% sucrose, 5 µM ABA and 0.8% agar

(maturation medium) was adopted for this purpose. Microscopic observation was conducted to ensure the development of somatic embryos. The total number of somatic embryos produced after 2 weeks on maturation medium per one gram fresh weight of embryogenic tissue is summarized in Table-2. At first, somatic embryos appeared as small white to yellow protuberances on the surface of the callus (Figure 1.3). Furthermore, we never observed embryogenesis without callus formation. It was also observed that frequency of somatic embryo formation was higher in white to yellowish, friable, and crystal calli than in watery, loose ones. The embryos passed through recognizable globular, heart, torpedo and early cotyledonary stages, finally resulting in germinated embryos on the germination medium (Fig 1.4). The callus developed somatic embryos on maturation medium after a period of 2 weeks (Fig-1.3). The percentage of somatic embryogenesis was not similar in all the tested varieties of *S. officinarum* (Table-2).Genotypic influence on somatic embryogenesis has been observed previously and a similar explanation holds for the differential response between the cultivars tested by us. After maturation, somatic embryos were picked from the cultures for germination (Fig-1.4). The germination medium used was half strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium with 0.7% agar without any growth regulators (germination medium). A large number of somatic embryos were continuously developed and germinated with a distinct shoot meristem and radicular end (Figu-1.4).

Table 1: The effect of various concentrations of TDZ on initiation of embryogenic tissue in different varieties of sugarcane cultured on full strength MS basal medium supplemented with 4.52μM 2, 4-D

TDZ con (μM)	Embryogenic tissue initiation in different sugarcane varieties (%)					
	Co671*	Co86032*	Co94032*	SNK754*	SNK61*	SNK44*
control	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c
0.45	4.0±0.1b	1.0±0.2b	1.0±0.1b	3.0±0.2b	1.0±0.1b	2.0±0.1b
2.27	17.0±0.3b	8.0±0.2b	11.0±0.5b	15.0±0.6b	3.0±0.4b	5.0±0.2b
4.54	71.0±4.5a	85.0±3.0a	58.0±1.5a	36.0±0.9a	29.0±0.5a	60.0±0.6a
9.08	3.0±0.1b	2.0±0.3b	0.0±0.0c	0.0±0.0c	3.0±0.3b	1.0±0.1b
11.35	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c	0.0±0.0c

Control=MS basal medium without growth regulators such as 2, 4-D and TDZ; Data scored after 8 weeks and represents the mean ±SE of at least 3 different experiments. In each column, the values with different letters are significantly different (P<0. 5). *Different sugarcane varieties.

Hawaiian researchers, the pioneers of sugarcane tissue culture (Nickell, 1964), reported the first successful plant regeneration from sugarcane callus cultures (Barba and Nickell, 1969; Heinz and Mee, 1969; Chen *et al*. 1988). Induction of somatic embryogenesis and plant regeneration has been reported successfully in sugarcane (Ho and Vasil, 1983a, b). Ahloowalia and Maretzki, (1983) were the first to provide convincing evidence of plant regeneration through somatic embryogenesis in sugarcane. Plant regeneration from sugarcane callus cultures has long been established. In callus cultures, the mode of plant regeneration is reported to be through SE (Liu and Chen, 1974; Nadar *et al*. 1977; Zeng, 1979; Liu, 1993; Srinivasan and Vasil, 1986; Gallo-Meagher *et*

al. 2000). In another study, a protocol for direct somatic embryogenesis using immature inflorescence segments as the explants of sugarcane has been reported (Desai *et al*. 2004; Suprasanna *et al*. 2008).

Table 2: Somatic embryogenesis and seedling recovery in different varieties of *Saccharam officinarum*

Sugarcane varieties	Somatic embryogenesis (%)	Somatic embryos recovered per gram fresh wt of Embryogenic tissue	Seedlings recovered per gram fresh wt of embryogenic tissue
CoC671*	71.0±4.5a	41.0±2.5a	21.0±2.0a
control	0.0±0.0b	0.0±0.0b	0.0±0.0b
Co86032*	85.0±3.0a	63.0±4.5a	36.0±2.9a
control	0.0±0.0b	0.0±0.0b	0.0±0.0b
Co94032*	58.0±1.5a	23.0±0.5a	13.0±0.2a
control	0.0±0.0b	0.0±0.0b	0.0±0.0b
SNK754*	36.0±0.9a	16.0±0.4a	9.0±0.4a
control	0.0±0.0b	0.0±0.0b	0.0±0.0b
SNK61*	29.0±0.5a	20.0±0.8a	8.0±0.3a
control	0.0±0.0b	0.0±0.0b	0.0±0.0b
SNK44*	60.0±0.6a	37.0±0.2a	22.0±0.1a
control	0.0±0.0b	0.0±0.0b	0.0±0.0b

Control=MS basal medium without growth regulators such as 2, 4-D and TDZ; Data scored after 6 weeks and represents the mean ±SE of at least 3 different experiments. In each column, the values with different letters are significantly different (P<0. 5). *Different sugarcane varieties.

Recently TDZ induction of somatic embryogenesis and plant regeneration has been successfully reported in grapes (*V. vinifera*) (Malabadi *et al.* 2010) and mango (*M. indica*) (Malabadi *et al*. 2011a). TDZ is a substituted phenyl urea with cytokinin-like activity and therefore, stimulates rapid shoot differentiation (Mok *et al.* 1982). TDZ aids in rapid plant regeneration of a number of plant species through organogenesis (Malik and Saxena, 1992). A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* cv. 'Chiengmai Pink' was developed using TDZ (Chung *et al.* 2007). 5-25% of leaf tip segments of *in vitro*-grown plants directly formed somatic embryos on half-strength MS medium supplemented with 0.3, 1 and 3.0 mg/l TDZ (Chung *et al.* 2007). Recently Chhabra *et al.* (2008) reported that TDZ at concentration lower than 2.0 µM induced shoot organogenesis whereas at higher concentration (2.5-15 µM) it caused a shift in regeneration from shoot organogenesis to somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.). TDZ at 0.5 and 5.0 µM was found to be optimal for inducing an average of 4-5 shoots per cotyledonary node in 93 % of the cultures and 55 somatic embryos in 68 % of the cultures of lentil (*Lens culinaris* Medik.) (Chhabra *et al*. 2008). The somatic embryos were germinated when transferred to lower TDZ concentration (0.5-1.0 µM) in lentil (*Lens culinaris* Medik.) (Chhabra *et al*. 2008). The rapid direct and repetitive somatic embryogenesis in *Coffea arabica* and *C. canephora* genotypes was tested on MS

containing thidiazuron (TDZ) (1-phenyl-3-(1,2,3,-thiadiazol-5-yl)urea) in concentrations of 2.27–11.35s µM (Giridhar *et al.* 2004). Segments taken from cotyledon leaf, first leaf and stalk of regenerated plantlets produced clusters of somatic embryos directly from cut portions of explants on TDZ (9.08 µM) containing medium within a period of two months. Subculturing of these embryo clusters produced more secondary embryos on reduced TDZ (0.045–0.91 µM) containing medium and these subsequently developed into plantlets (80–85%) on development medium followed by rooting on MS basal medium (Giridhar *et al.* 2004).

In *Costus speciosus*, rhizome thin sections cultured on B_5 basal medium without TDZ (control) or with low concentrations 0.45 and 4.54 µM TDZ completely failed to produce shoot buds. Higher concentrations of TDZ, particularly 36.32, 40.86 and 45.41 µM, resulted in the browning of explants which finally necrosed (Malabadi *et al.* 2004b). On the other hand, initiation of shoot buds was observed in the range 11.35-27.34 µM TDZ with highest percentage of rhizome thin sections (92%) producing shoot buds (12 ± 2.01) at 18.16 µM TDZ (Malabadi *et al.* 2004b). A sharp decrease in the number of shoot bud formation was also noticed when the concentration of TDZ was increased from 18.16 to 31.78 µM (Malabadi *et al*. 2004b). Least and poor growth of shoot buds (1.8 ± 0.02) was noticed at 31.78 µM TDZ in *Costus speciosus* (Malabadi *et al.* 2004b). An efficient shoot regeneration of *Eria dalzelli* (Dalz.) Lindl. for the first time was achieved using shoot tip tTCLs and TDZ (Malabadi *et al*. 2008a). As in this study, PLBs or proliferating shoot buds were observed when shoot tip tTCLs were cultured on the same basal medium supplemented with 9.08 µM TDZ. The highest percentage (96%) of PLBs survived and ultimately produced healthy shoots with 2-3 leaves (Malabadi *et al*. 2008a). An efficient shoot regeneration of *Vanda coerulea* was achieved using TCLs and TDZ (Malabadi *et al.* 2004a).

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